

***Staphylococcus epidermidis* biofilms control by N-acetylcysteine and rifampicin**

*Bruna Leite^{a,b}, MSc, Fernanda Gomes^a, BSc, Pilar Teixeira^a, PhD, Clovis Souza^b, PhD, Elisabeth Pizzolitto^{b,c}, PhD, Rosário Oliveira^a, PhD**

^aIBB-Institute for Biotechnology and Bioengineering, University of Minho, Campus of Gualtar, Braga, Portugal.

^bDepartment of Biotechnology, Federal University of São Carlos, São Carlos, Brazil.

^cFaculty of Pharmaceutical Sciences, São Paulo State University, Araraquara, Brazil.

*Correspondence to: Rosário Oliveira, IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal; Tel: +351253604409; Fax: +351253678986; E-mail: roliveira@deb.uminho.pt

Running title: *N*-acetylcysteine as therapeutic adjuvant

24

25 **Abstract**

26 Medical device-associated infections caused by *Staphylococcus*
27 *epidermidis* usually involve biofilm formation and its eradication is
28 particularly challenging. Although rifampicin has been proving to be one of
29 the most effective antibiotics against *S. epidermidis* biofilms its use as a
30 single agent can lead to the acquisition of resistance. Therefore, we
31 assessed the combined effect of rifampicin with *N*-acetylcysteine (NAC)
32 known by its mucolytic effect, in the control of *S. epidermidis* biofilms.
33 Biofilms of two *S. epidermidis* strains (9142 and 1457) were treated with
34 1xMIC (4 mg/mL) and 10xMIC (40 mg/mL) of NAC and 10 mg/L (peak
35 serum) of rifampicin alone and in combination. NAC at 40 mg/L alone or in
36 combination with rifampicin (10 mg/L) significantly reduced (4 log₁₀) the
37 number of biofilm cells. Considering their different modes of action, the
38 association of NAC with rifampicin constitutes a promising therapeutic
39 strategy in the treatment of infections associated to *S. epidermidis*
40 biofilms.

41

42

43 **Key words:** *Staphylococcus epidermidis*, biofilm, planktonic cells, *N*-
44 acetylcysteine, rifampicin.

45

46

48 INTRODUCTION

49 *Staphylococcus epidermidis* and other coagulase-negative
50 staphylococci (CoNS) produce extracellular matrix, which is an important
51 virulence factor. This polymeric matrix promotes bacterial adhesion and
52 produces a biofilm that makes the eradication of microorganisms more
53 difficult (1). *Staphylococcus epidermidis*, the most frequently isolated
54 coagulase-negative staphylococcus, is the leading cause of infection
55 related to implanted medical devices (IMDs). This is directly related to its
56 capability to establish multilayered, highly structured biofilms on artificial
57 surfaces. Bacterial biofilms are difficult to detect in routine diagnostics and
58 are inherently tolerant to host defenses and conventional antibiotic
59 therapies. Thus, device-related infections are notoriously difficult to treat
60 and bacteria within biofilm communities on the surface of IMDs frequently
61 outlive treatment, and removal of the medical device is often required for
62 successful therapy (2). Additionally, the emergence of antibiotic-resistant
63 bacteria and the slow progress in identifying new classes of antimicrobial
64 agents have encouraged research into novel therapeutic strategies (3).

65 *N*-acetylcysteine (NAC) is a non-antibiotic drug that has antibacterial
66 properties. It is a mucolytic agent that disrupts disulphide bonds in mucus
67 and reduces the viscosity of secretions (4). NAC is commonly used in
68 medical treatment of chronic bronchitis and cancer (5, 6) and is one of the
69 smallest drug molecules in use (7). NAC affects several processes that are

1
2 70 important for bacterial biofilm formation on stainless steel surfaces,
3
4 71 including a drastic reduction in extracellular polysaccharide production,
5
6
7 72 and thus acts as an antibiofilm substance (8).
8
9

10 73 Previous results have demonstrated that rifampicin is the most
11
12 74 effective of traditional antibiotics against *S. epidermidis* cells in biofilms (9-
13
14 75 12).
15

16
17 76 Accordingly, the aim of this work was to determine the *in vitro* effect
18
19 77 of NAC alone but specifically in combination with rifampicin on biofilms of
20
21
22 78 two different *S. epidermidis* strains.
23

24 79

25
26 80

27
28
29 81

30
31 82

32
33
34 83

35
36 84

37
38
39 85

40
41 86

42
43
44 87

45
46 88

47
48
49 89

50
51 90

52
53
54 91

55
56 92

93

94 **MATERIALS AND METHODS**

95 **Bacterial strains and growth conditions**

96 Two *Staphylococcus epidermidis* clinical isolate strains (9142 and
97 1457, good biofilm-producers) were used in this study. Both strains were
98 provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine,
99 Brigham and Women's Hospital, Harvard Medical School, Boston, USA.

100 The culture media used tryptic soy broth (TSB) and tryptic soy agar
101 (TSA), were prepared according to the manufacturer's instructions. All
102 strains were inoculated into 15 mL of TSB from TSA plates not older than
103 2 days and grown for 24 (± 2) h at 37°C in an orbital shaker at 130 rpm.
104 Cells were harvested by centrifugation (for 5 min at 9500g and 4°C), and
105 resuspended in TSB adjusted to an optical density (640 nm) equivalent to
106 1×10^6 cells/mL and then used in the subsequent assays.

107 Solutions of NAC 4 mg/mL (1x MIC) and 40 mg/mL (10x MIC) (13)
108 were prepared in TSB and stock solution of rifampicin (10 mg/L - peak
109 serum concentration) (9, 14) was prepared in methanol.

110

111 **Minimum inhibitory concentration (MIC)**

112 MIC determination of the tested agent *N*-acetylcysteine and for each
113 *S. epidermidis* strain (9142 and 1457) was carried with dilution range of
114 0.5-64 mg/mL.

1
2
3 115 The MIC was determined in 96 well tissue culture plates (Sarstedt,
4
5 116 Newton, NC, USA) containing 100 μ L of a stock solution of NAC (64
6
7 117 mg/mL) and the dilution was realized with TSB, adding at the end 100 μ L
8
9 118 of a *S. epidermidis* cell suspension (1×10^6 cells/mL). Plates were
10
11 119 incubated at 37°C with orbital shaking at 130 rpm for 24 h. After
12
13 120 incubation the minimum inhibitory concentration was determined with the
14
15 121 lowest concentration able to inhibit bacterial growth.
16
17
18

19 122 The controls were not exposed to the antimicrobial agent tested. All
20
21 123 experiments were carried out in triplicate and repeated three times.
22
23
24

25 124

26 125 **Effect of NAC and rifampicin on biofilm cells**

27
28
29 126 Biofilms were formed in 96 well tissue culture plates (Sarstedt,
30
31 127 Newton, NC, USA) containing 200 μ L of a *S. epidermidis* cell suspension
32
33 128 (1×10^6 cells/mL) in TSB supplemented with 0.25% of glucose per well to
34
35 129 promote biofilm formation. Plates were incubated at 37°C with orbital
36
37 130 shaking at 130 rpm for 24 h. At the end, planktonic cells were removed
38
39 131 carefully, and the biofilm was washed twice with 200 μ L of saline solution
40
41 132 (0.9% NaCl; Merck). The biofilms were incubated for 24 h in fresh nutrient
42
43 133 medium containing NAC (4 mg/mL and 40 mg/mL) or rifampicin (10 mg/L)
44
45 134 alone or a combination of both NAC concentrations tested with rifampicin.
46
47
48
49
50

51 135 Crystal violet (CV) staining was used as indicator of total biofilm
52
53 136 biomass. After exposure to the treatment agents, biofilms were washed
54
55 137 with 200 μ L of saline solution, then 250 μ L of methanol was added and
56
57
58
59
60
61
62
63
64
65

1
2 138 allowed to act for 15 min. Afterwards methanol was removed and crystal
3
4 139 violet was added (5 min). The wells were washed with water and finally,
5
6
7 140 acetic acid 33% (v/v) was added. The absorbance was measured at 570
8
9 141 nm.

10
11
12 142 Another colorimetric method based on the reduction of XTT ({2,3-
13
14 143 bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phe-nylamino)carbonyl]-2H-
15
16
17 144 tetrazolium hydroxide}; Sigma, St Louis, USA) was applied to determine
18
19 145 cell activity (XTT is converted to a colored formazan salt in the presence of
20
21 146 metabolic activity) (15). After exposure to antimicrobial agents, biofilms
22
23
24 147 were washed with 200 µL of saline solution, then 200 µL of a solution
25
26 148 containing 200 mg/L of XTT and 20 mg/L of phenazine methosulphate
27
28 149 (PMS; Sigma, St Louis, USA) was added to each well. The microtiter
29
30
31 150 plates were incubated for 3 h at 37°C in the dark. The absorbance was
32
33
34 151 measured at 490 nm.

35
36 152 To assess the number of viable cells after treatment, 200 µL of saline
37
38
39 153 solution were added to each well before removing the biofilm by
40
41 154 scrapping. An aliquot of 1 mL of each sample was centrifuged (for 10 min
42
43 155 at 9500g and 4°C) and the pellet resuspended in 1 mL of saline solution.
44
45
46 156 Next, the suspension was sonicated (20 s with 22% of amplitude;
47
48
49 157 Ultrasonic Processor, Cole-Parmer, Illinois, USA) to promote biofilm
50
51 158 disruption. The number of colony forming units (CFU) in biofilm for each *S.*
52
53 159 *epidermidis* strain was determined by performing 10-fold serial dilutions in
54
55
56 160 saline solution and plating in TSA in triplicate and incubating for 24–48 h.

161 Biofilm controls were not exposed to any antimicrobial agent tested.

162 All experiments were carried out in triplicate and repeated three times.

163 **Effect of NAC and rifampicin on planktonic cells**

164 For each strain, 200 μ L of a cell suspension adjusted to 1×10^9
165 cells/mL and 1.5 mL of that suspension was added to 15 mL of TSB until a
166 cell density of 1×10^8 cells/mL was reached and were then dispensed in
167 test tubes followed by the addition of the treating agents alone, NAC (4
168 mg/mL and 40 mg/mL) or rifampicin (10 mg/L), and in combination. All the
169 tubes were incubated at 37°C with shaking at 130 rpm.

170 Cell susceptibility to the treatment agents was determined by the
171 XTT colorimetric method. For that, 1 mL of each sample was collected and
172 centrifuged (for 10 min at 9500g and 4°C) and the pellet was
173 resuspended in 1 mL of saline solution (0.9% NaCl; Merck). From each
174 suspension, 200 μ L were transferred to individual wells of a 96-well
175 microtiter plate. Then, 50 μ L of a solution containing 200 mg/L of XTT and
176 20 mg/L of PMS were added to each well and the microtiter plates were
177 incubated for 3 h at 37°C in the dark. The absorbance was measured at
178 490 nm.

179 For CFU determination, 1 mL of each sample was collected and
180 centrifuged (for 10 min at 9500g and 4°C) and the pellet resuspended in
181 1 mL of saline solution. The suspension was sonicated (20 s with 22% of
182 amplitude; Ultrasonic Processor, Cole-Parmer, Illinois, USA) and the
183 viable planktonic cells were determined by performing 10-fold serial

184 dilutions in saline solution and plating in TSA. Colonies were counted after
185 24-48 h at 37°C.

186 Controls were planktonic cells not exposed to any treatment agent
187 tested. All experiments were carried out in triplicate and repeated three
188 times.

190 **Extraction of the exopolymeric matrix**

191 Biofilms were formed in 6 well tissue culture plates (Sarstedt,
192 Newton, NC, USA) containing 4 mL of a *S. epidermidis* cell suspension (1×10^6 cells/mL) in TSB supplemented with 0.25% of glucose per well to
193 promote biofilm formation. Plates were incubated at 37°C with orbital
194 shaking at 130 rpm for 24 h. At the end, planktonic cells were removed
195 carefully, and the biofilm was washed twice with 4 mL of saline solution.
196 The biofilms were incubated in fresh nutrient medium containing NAC (4
197 mg/mL and 40 mg/mL), rifampicin (10 mg/L) alone and NAC in
198 combination with rifampicin. After 24 h of incubation, the biofilm was
199 washed twice with 4 mL of saline solution. Next, 4 mL of phosphate
200 buffered saline (pH 7.0) were added and the biofilm formed was removed
201 from the well by scrapping with a cell scraper (zellschaber/24 cm). The
202 samples were centrifuged for 5 min at 9500g and 4°C, and resuspended
203 in 5 mL of extraction buffer (2 mM Na₃PO₄; 4 mM NaH₂PO₄; 9 mM NaCl
204 and 1 mM KCl, pH 7.0) and 1 g of resin (Dowex/50X8, Na⁺ form, 20-50
205 mesh, Aldrich-Fluka 44445) was added. The samples were incubated at -

207 5°C for 2 h. After the incubation the samples were centrifuged for 20 min
208 at 9500g and 4°C.

209 The polysaccharide content in the biofilm matrix was quantified by
210 the method of Dubois et al. (16). The protein content of the biofilm matrix
211 was measured using the BCAtm Protein Assay Kit (Bicinchoninic Acid,
212 Sigma-Aldrich, St Louis, USA).

213 Controls were biofilms not exposed to any treatment agent tested. All
214 experiments were carried out in triplicate and repeated three times.

215

216 **Scanning electron microscopy (SEM)**

217 Biofilms formed in 12 well tissue culture plates (Sarstedt, Newton,
218 NC, USA) were dehydrated by 15 min immersion in increasing ethanol
219 concentrations (70, 95 and 100% [v/v]), and then placed in a sealed
220 desiccator. The samples were mounted on aluminum stubs with carbon
221 tape, sputter coated with gold and observed with a Leica Cambridge S-
222 360 scanning electron microscope (Leo, Cambridge, UK).

223 Controls were biofilms not exposed to any treatment agent tested. All
224 experiments were carried out in triplicate.

225

226 **Statistical Analysis**

227 The data from all assays were compared using one-way analysis of
228 variance (ANOVA) by applying Tukey's test with all calculations carried out
229 using SPSS software (Statistical Package for the Social Sciences).

230 Differences achieving a confidence level of 95% were considered
231 significant.

232 RESULTS

233 Figure 1 presents the number of viable cells of the two *S. epidermidis*
234 strains tested, expressed as log CFU, remaining after the treatment of
235 either biofilm or planktonic cells. It can be observed that NAC alone at 4
236 mg/mL (1x MIC) had a slight inhibitory effect on planktonic cells but almost
237 no effect on biofilms, while at 10x MIC it showed a notorious killing effect
238 on planktonic cells (almost total eradication) and a significant bactericidal
239 effect on biofilms, in this case promoting CFU reductions of about 4 log₁₀
240 (p<0.05). Rifampicin alone, showed an effect on biofilm cells promoting
241 CFU reduction of about 3 log₁₀ (p<0.05). Although the reduction promoted
242 by rifampicin in planktonic cells was less than 3 log. The NAC-rifampicin
243 combination consistently decreased the number of viable biofilm
244 associated bacteria by 3-4 log₁₀ independently of NAC concentration used.
245 In fact, NAC at 40 mg/ml (10x MIC), rifampicin alone or NAC-rifampicin
246 combinations showed significant but similar bactericidal effect against
247 biofilms (p<0.05). The combination of NAC (10x MIC) with rifampicin in
248 planktonic cells promoted a killing effect (p<0.05).

249 The results expressing the decrease in metabolic activity measured
250 by the XTT reduction assay after treatment with the tested agents are
251 showed in figure 2.

252 In addition, total biofilm biomass, assessed by CV staining, also
253 confirms the effect of the agents tested as can be seen in figure 3.

254 The matrix composition showed a generally significant increase
255 ($p<0.05$) in the amount of proteins and polysaccharides after treatment,
256 presented in figure 4. The amount of proteins was very high after
257 treatment with the combination NAC 40 mg/mL with rifampicin ($p<0.05$).
258 However, the amount of polysaccharides was high after treatment with
259 NAC (10x MIC) alone or the combination NAC-rifampicin, independently of
260 NAC concentration used ($p<0.05$).

261 Scanning electron microscope images of the biofilms are presented
262 in figure 5. The SEM shows representative images of *S. epidermidis*
263 (strain 1457) biofilms after treatment with the agents tested alone and in
264 combination. As can be seen (Figure 5C and F), after treatment with NAC
265 at 40 mg/mL alone or combined with rifampicin the amount of matrix
266 present is very small and this might be due to an easy removal of the
267 matrix during the desiccation procedure by successively washing with
268 increasing ethanol concentrations.

269

270

271

272

273

274

275

276 **DISCUSSION**

277 Modern medicine is facing the challenge to control the increasing
278 incidence of biofilm infections and this situation has boosted the search of
279 new therapeutic strategies able to evade the intrinsic tolerance of biofilms
280 to antimicrobial agents. Accordingly, the rationale of this study stems from
281 the reported effect of NAC in disrupting mature biofilms (4) and the high
282 efficacy of rifampicin against *S. epidermidis* biofilms when compared with
283 other common antibiotics (9-12). Thus, we hypothesized that in
284 combination they could have a synergistic effect due to their different
285 modes of action.

286 The great efficacy of NAC against planktonic cells was also
287 confirmed in experiments with other gram-positive and gram-negative
288 strains, showing that NAC did indeed reduce the growth of all strains
289 tested (8). Rifampicin alone, as we could expect from previous studies (9-
290 11), showed higher efficacy against biofilm cells than planktonic cells. As
291 demonstrated by viable cells reduction (Figure 1) and in opposition to our
292 hypothesis the combinations tested were not synergistic or additive in
293 controlling *S. epidermidis* biofilms. In a previous study (13), reported a
294 synergistic effect of NAC in combination with tigecycline on *S. epidermidis*
295 biofilms using NAC at 20x MIC (80 mg/mL) and tigecycline at 1000x MIC
296 (1 mg/mL) with the MIC values reporting to planktonic cells. Nevertheless,

1
2 297 their results in viable cells reduction of this species were less than 3 log₁₀.
3
4 298 Regarding the NAC-rifampicin combination tested herein the values
5
6
7 299 obtained where higher, corresponding to a bactericidal action, even if not
8
9
10 300 corresponding to a synergy between the two agents.

11
12 301 In this work, the biofilm formation capability was as well evaluated
13
14 302 through crystal violet, allowing the quantification of the total biomass. The
15
16
17 303 metabolic activity of biofilms was evaluated through XTT reduction assays.
18
19 304 The results shown are in very good agreement with those obtained in
20
21
22 305 terms of cell viability.

23
24 306 Interestingly, the matrix composition showed a generally significant
25
26 307 increase in the amount of proteins and polysaccharides after treatment
27
28
29 308 (Figure 4). Notoriously, the amount of proteins was very high after
30
31
32 309 treatment, which is consistent with a higher degree of cell lysis. The
33
34 310 increase in polysaccharides content is probably due in greater extent to
35
36 311 the loosening effect of NAC on the matrix structure and thus making its
37
38
39 312 extraction more efficacious. As shown previously the strain 1457 produces
40
41 313 more extracellular polysaccharides than 9142 (17). Moreover, NAC is a
42
43 314 thiol containing molecule that is described to disrupt disulfide bonds in
44
45
46 315 mucus (18). In fact, SEM observations support this hypothesis.

47
48 316 It should be stressed that it is now generally accepted that a
49
50
51 317 combination of antimicrobial agents is a strategy to evade the
52
53
54 318 development of bacterial resistance to antibiotics. In fact, it is not
55
56 319 advisable to use rifampicin as a single agent to treat infections because of

the rapid selection of resistant mutants (19). Zheng and Stewart (20) detected rifampicin-resistant mutants, when colony biofilms were exposed to rifampicin for periods longer than 48 h. In the present case, the combination 40 mg/mL NAC- 10 mg/L rifampicin showed a higher and real bactericidal effect on biofilms (above 3 log₁₀), which probably opens the way for an efficient action of the immune system in the eradication of *in vivo* biofilms.

343

344

345 **ACKNOWLEDGEMENTS**

346 The author acknowledges the financial support of ISAC/ Program
347 Erasmus Munds External Cooperation and the IBB-Institute for
348 Biotechnology and Bioengineering, Centre of Biological Engineering,
349 University of Minho, Campus of Gualtar.

350

351

352

353

354

355

356

357

358

359

360

361

362

363

364

365

366

367

368 REFERENCES

- 369 1. Kloos WE, Bannerman TL. Update on clinical significance of
370 coagulase-negative staphylococci. *Clin Microbiol Rev.* 1994;7(1):117-140.
- 371 2. McCann MT, Gilmore BF, Gorman SP. *Staphylococcus epidermidis*
372 device-related infections: Pathogenesis and clinical management. *Journal*
373 *of Pharmacy and Pharmacology.* 2008;60:1551-1571.
- 374 3. Costerton W, Montanaro L, Balaban N, et al. Prospecting gene
375 therapy of implant infections. *Int J Artif Organs.* 2009;32(9):689-695.
- 376 4. Perez-Giraldo C, Rodriguez-Benito A, Moran FJ, et al. Influence of
377 n-acetylcysteine on the formation of biofilm by *Staphylococcus*
378 *epidermidis.* *J Antimicrob Chemother.* 1997;39(5):643-646.
- 379 5. Rehm BHA, Valla S. Bacterial alginates: Biosynthesis and
380 applications. *Applied Microbiology and Biotechnology.* 1997;48(3):281-
381 288.
- 382 6. Stey C, Steurer J, Bachmann S, et al. The effect of oral n-
383 acetylcysteine in chronic bronchitis: A quantitative systematic review. *Eur*
384 *Respir J.* 2000;16(2):253-262.
- 385 7. Noszal B, Visky D, Kraszni M. Population, acid-base, and redox
386 properties of n-acetylcysteine conformers. *Journal of Medicinal Chemistry.*
387 2000;43(11):2176-2182.

- 1
2 388 8. Olofsson A-C, Hermansson M, Elwing H. N-acetyl-L-cysteine affects
3
4 389 growth, extracellular polysaccharide production, and bacterial biofilm
5
6 390 formation on solid surfaces. *Appl Environ Microbiol*. 2003;69(8):4814-
7
8 391 4822.
9
10
11 392 9. Cerca N, Martins S, Cerca F, et al. Comparative assessment of
12
13 393 antibiotic susceptibility of coagulase-negative staphylococci in biofilm
14
15 394 versus planktonic culture as assessed by bacterial enumeration or rapid
16
17 395 xtt colorimetry. *J Antimicrob Chemother*. 2005;56(2):331-336.
18
19
20 396 10. Monzon M, Oteiza C, Leiva J, et al. Synergy of different antibiotic
21
22 397 combinations in biofilms of staphylococcus epidermidis. *J Antimicrob*
23
24 398 *Chemother*. 2001;48(6):793-801.
25
26
27 399 11. Svensson E, Hanberger H, Nilsson M, et al. Factors affecting
28
29 400 development of rifampicin resistance in biofilm- producing *Staphylococcus*
30
31 401 *epidermidis*. *J Antimicrob Chemother*. 1997;39(6):817-820.
32
33
34 402 12. Svensson E, Hanberger H, Nilsson LE. Pharmacodynamic effects
35
36 403 of antibiotics and antibiotic combinations on growing and nongrowing
37
38 404 *Staphylococcus epidermidis* cells. *Antimicrob Agents Chemother*.
39
40 405 1997;41(1):107-111.
41
42
43 406 13. Aslam S, Trautner BW, Ramanathan V, et al. Combination of
44
45 407 tigecycline and n-acetylcysteine reduces biofilm-embedded bacteria on
46
47 408 vascular catheters. *Antimicrob Agents Chemother*. 2007;51(4):1556-1558.
48
49
50 409 14. Conte JE, Lin E, Zurlinden E. High-performance liquid
51
52 410 chromatographic determination of pyrazinamide in human plasma,
53
54
55
56
57
58
59
60
61
62
63
64
65

411 bronchoalveolar lavage fluid, and alveolar cells. *Journal of*
 412 *Chromatographic Science*. 2000;38:33-37.
 413 15. Kuhn DM, Balkis M, Chandra J, et al. Uses and limitations of the xtt
 414 assay in studies of candida growth and metabolism. *J Clin Microbiol*.
 415 2003;41(1):506-508.
 416 16. DuBois M, Gilles KA, Hamilton JK, et al. Colorimetric method for
 417 determination of sugars and related substances. *Analytical Chemistry*.
 418 1956;28(3):350-356.
 419 17. Sousa C, Teixeira P, Oliveira R. The role of extracellular polymers
 420 on *Staphylococcus epidermidis* biofilm biomass and metabolic activity.
 421 *Journal of Basic Microbiology*. 2009;49(4):363-370.
 422 18. Sheffner AL. The reduction *in vitro* in viscosity of mucoprotein
 423 solutions by a new mucolytic agent, n-acetyl-l-cysteine. *Annals of the New*
 424 *York Academy of Sciences*. 1963;106:298-310.
 425 19. Mick V, Dominguez MA, Tubau F, et al. Molecular characterization
 426 of resistance to rifampicin in an emerging hospital-associated methicillin-
 427 resistant *Staphylococcus aureus* clone st228, spain. *BMC Microbiology*.
 428 2010;10(1):68.
 429 20. Zheng Z, Stewart PS. Penetration of rifampin through
 430 *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother*.
 431 2002;46(3):900-903.
 432
 433

434

435

436 **LEGEND TO FIGURES**

437

438 Figure 1. Number of viable cells (expressed as log₁₀ CFU) of *S.*
439 *epidermidis* recovered from biofilm cells (A) and planktonic cells (B) after
440 treatment with NAC and rifampicin (RIF) alone or in combination.

441

442 Figure 2. Cellular activity expressed as XTT absorbance of *S.*
443 *epidermidis* biofilm cells (A) and planktonic cells (B) after treatment with
444 NAC and rifampicin (RIF) alone or in combination.

445

446 Figure 3. *S. epidermidis* total biofilm biomass expressed as crystal violet
447 absorbance after treatment with NAC and rifampicin (RIF) alone or in
448 combination on biofilm cells.

449

450 Figure 4. Quantification of protein (A) and polysaccharides (B) of the
451 exopolymeric matrix of *S. epidermidis* (9142 and 1457) biofilms.

452

453 Figure 5. Scanning electron microscopy photographs of 24 h *S.*
454 *epidermidis* (strain 1457) biofilms: control (A), NAC 4 mg/mL (B), NAC 40
455 mg/mL (C), rifampicin (D), NAC (4 mg/mL) with rifampicin (E) and NAC

1
2 456 (40 mg/mL) with rifampicin (F). The bar in the images corresponds to
3
4
5 457 5µm. Magnification x 5000.
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65